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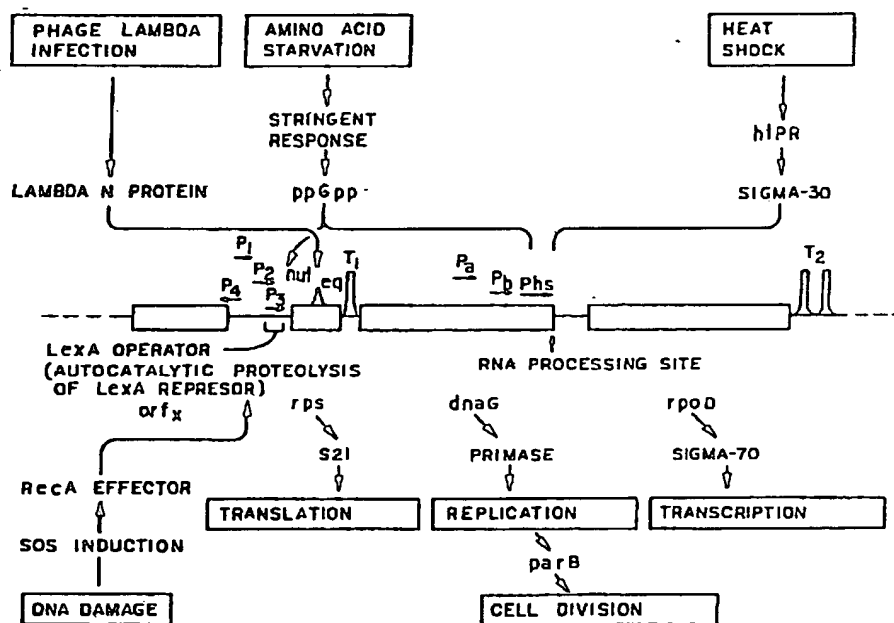
INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁴ : C12Q 1/68, C12N 15/00 C07H 15/12		A1	(11) International Publication Number: WO 90/00624
			(43) International Publication Date: 25 January 1990 (25.01.90)
(21) International Application Number: PCT/US89/02884		SE (European patent).	
(22) International Filing Date: 30 June 1989 (30.06.89)		Published	
(30) Priority data: 215,135 5 July 1988 (05.07.88) US		<i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
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(81) Designated States: AT (European patent), AU, BE (European patent), CH (European patent), DE (European patent), FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent),			

(54) Title: ANTISENSE OLIGONUCLEOTIDE ANTIBIOTICS COMPLEMENTARY TO THE MACROMOLECULAR SYNTHESIS OPERON, METHODS OF TREATING BACTERIAL INFECTIONS AND METHODS FOR IDENTIFICATION OF BACTERIA

(57) Abstract

A method of interrupting the expression of a macromolecular synthesis operon in bacteria comprising the step of binding an antisense oligonucleotide to a single stranded DNA or to a mRNA transcribed from the macromolecular synthesis operon. The antisense oligonucleotide can be either sequence specific to a unique intergenic sequence or a sequence specific to a bacterial homologous sequence. By interrupting the expression of the macromolecular synthesis operon bacterial infections can be treated. Examples of antisense oligonucleotides are 5'CATCCAAG-CAGTGGTAAACTGTTT 3', 5'TCACCGATCGGCGTTTCCA 3', 5'GGCCCCGATTTTAGCAA 3', 5'CTTGCGTA-ACGCCGGGG 3', and 5'TATTCGATGCTTTAGTGC 3'. The ability of the antisense oligonucleotide to bind the mRNA or single stranded DNA also allows the identification of the bacteria by using a unique intergenic antisense oligonucleotide to bind to the single stranded DNA or the mRNA transcribed from the macromolecular synthesis operon. A method for competitively inhibiting the protein products of the MMS operon with oligonucleotides is also disclosed.



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ANTISENSE OLIGONUCLEOTIDE ANTIBIOTICS
COMPLEMENTARY TO THE MACROMOLECULAR
SYNTHESIS OPERON, METHODS OF TREATING
BACTERIAL INFECTIONS
AND METHODS FOR IDENTIFICATION OF BACTERIA

15

FIELD OF THE INVENTION

The present invention relates generally to
antisense oligonucleotides which bind to a messenger RNA.
More particularly it relates to antisense oligonucleotides
which bind to messenger RNA transcribed from the
macromolecular synthesis operon of bacteria. It also
relates to the treatment of bacterial infections by the
introduction of antisense oligonucleotides into bacteria.
It further relates to the method of identification of
bacteria by the binding of an antisense oligonucleotide
specifically to a unique sequence in the intergenic
regions of the macromolecular synthesis operon of
bacteria. It also relates to the treatment of bacterial
infections by competitive inhibition of the macromolecular
synthesis operon gene products by utilizing
oligonucleotides known to act as recognition sequences for
the MMS operon protein products.

35

BACKGROUND OF THE INVENTION

It has been demonstrated that the genes involved in initiating the synthesis of DNA, RNA and protein in bacteria are contained in one single structural unit named the macromolecular synthesis operon (MMS). The genes are part of a single transcription unit and have been identified as rpsU encoding ribosomal protein S21 involved in initiating translation, dnaG encoding the protein primase which initiates DNA replication and rpoD which encodes sigma-70 involved in initiating transcription. The operon structure is found in both gram negative bacteria, such as Escherichia coli and Salmonella typhimurium, and in gram positive bacteria such as Bacillus subtilis. The individual structural genes are conserved and have large areas of homology. On the other hand, the intergenic sequences between the structural gene within the operon are unique to each bacterial species. The MMS operon appears to be a central information processing unit for directing the flow of genetic information. The organization of the operon suggests that under certain physiological conditions there is a need for coordination of synthesis of the information macromolecules (DNA, RNA and protein) in the cell and hence a coregulation of the initiator genes. Since the synthesis of each class of macromolecule appears to be regulated at its initiation step, regulation of the MMS operon most likely plays a role in regulating cell growth.

The MMS operon contains three structural genes. The rpsU gene encodes the ribosomal protein S21 which is required for specific initiation of messenger RNA (mRNA) translation. The protein S21 interacts with a stretch of ribosomal RNA (rRNA) complementary to the mRNA ribosomal binding site called the Shine-Dalgarno sequence located at the 3' end of the 16S rRNA. Colicin E3 removes 50

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1 nucleotides from the 3' terminus of 16S rRNA. E3 treated
ribosomes cannot carry out polypeptide chain initiation
nor chain elongation. In reconstitution experiments, E3
5 treated ribosomes bind all 30S proteins except S21. RNA
protein cross-linking experiments demonstrate that protein
S21 is cross-linked to the 3' dodecanucleotide of the 16S
rRNA. The base-pairing potential of the 3' terminus of
16S rRNA depends on the functional state of the 30S
10 subunit and the presence of S21, which is required for
specific initiation of E. coli and phage MS2 mRNA
translation.

Initiation of DNA replication requires a priming
RNA which is synthesized by the dnaG gene product,
15 primase. This protein binds to the phage G4 origin of
replication. Primase also is known to interact with the
multienzyme complex primosome to initiate synthesis of
Okazaki fragments on the chromosomal replication
fork-lagging strand of E. coli. Primase is the sole
20 priming enzyme required for initiation of DNA replication
at the origin of the E. coli chromosome. A parB mutation
in the dnaG gene results in abnormal partition of
chromosomes and was originally isolated as a
thermosensitive mutant affecting DNA synthesis and
25 cellular division. Thus, in addition to initiation of DNA
replication, the dnaG gene appears to play some role in
regulating cell division.

The rpoD gene product sigma-70 is involved in the
recognition of promoter sequences for the specific
30 initiation of RNA transcription. Sigma-70 interacts with
the core polymerase $\alpha_2\beta\beta'$ conferring specificity for
promoter sequences. Sigma-70 is a member of a large
family of RNA polymerase sigma factors. Thus, the
macromolecular synthesis operon gene products share a
common mechanism. Through protein-nucleic acid
35 interactions the gene products of the MMS operon bind

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1 specific nucleotide sequences. For example S21 binds the
Shine-Dalgarno sequence/ribosome binding site, primase
binds the origin of replication, and sigma-70 binds a
5 promoter sequence. These interactions result in
initiation of synthesis of protein, DNA or RNA
respectively.

Antisense RNAs have been utilized both in nature
and experimentally to regulate gene expression. For
10 example antisense RNA is important in plasmid DNA copy
number control, in development of bacteriophage P22.
Antisense RNAs have been used experimentally to
specifically inhibit in vitro translation of mRNA coding
from Drosophila hsp23, to inhibit Rous sarcoma virus
15 replication and to inhibit 3T3 cell proliferation when
directed toward the oncogene c-fos. Furthermore, it is
not necessary to use the entire antisense mRNA since a
short antisense oligonucleotide can inhibit gene
expression. This is seen in the inhibition of
20 chloramphenicol acetyltransferase gene expression and in
the inhibition of specific antiviral activity to vesicular
stomatitis virus by inhibiting the N protein initiation
site. Antisense oligonucleotides to the c-myc oncogene
have been demonstrated to inhibit entry into the S phase
25 but not the progress from G₀ to G₁. Finally,
inhibition of cellular proliferation has been demonstrated
by the use of antisense oligodeoxynucleotides to PCNA
cyclin.

Antibiotics are important pharmaceuticals for the
30 treatment of infectious diseases in a variety of animals
including man. The tremendous utility and efficacy of
antibiotics results from the interruption of bacterial
(prokaryotic) cell growth with minimal damage or side
effects to the eukaryotic host harboring the pathogenic
35 organisms. All antibiotics destroy bacteria by
interfering with the normal flow of genetic information.

1 This is performed by inhibition of any one of the
following: DNA replication, that is, DNA to DNA (for
example, the drugs Novobiocin and Nalidixic acid);
5 transcription, that is, DNA to RNA (for example,
Rifampin); translation, that is, RNA to protein (for
example, tetracyclines, erythromycin and kamanycin); or
cell wall synthesis (for example, penicillins).

The present invention provides a new class of
10 antibiotics and a method for the treatment of bacterial
infections either generally or specifically. The
antibiotics are antisense oligonucleotide sequences which
bind mRNA transcribed from the MMS operon. This is a new
method of treating bacterial infections by interfering
15 with the fundamental structural unit that regulates the
growth and replication of bacteria.

SUMMARY OF THE INVENTION

An object of the present invention is the
provision of a method for the treatment of bacterial
20 infections.

An additional object of the present invention is
the use of antisense oligonucleotides to treat bacterial
infections.

A further object of the present invention is a
25 method for identifying bacteria.

An additional object of the present invention is
the provision of antibiotics which interrupt the operation
of the macromolecular synthesis operon in bacteria.

A further object of the present invention is the
30 use of competitive inhibitors to interfere with the
nucleotide recognition site of the macromolecular operon
gene products.

Thus, in accomplishing the foregoing objects
there is provided in accordance with one aspect of the
35 present invention a method of interrupting the expression
of a MMS operon comprising the step of binding an

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1 antisense oligonucleotide to a mRNA transcribed from said
MMS operon. The antisense oligonucleotide sequence can be
specific to a unique intergenic sequence in the mRNA or it
5 can be a sequence which is specific to a region of the
mRNA containing a sequence which is homologous between
bacterial strains or any combination of these.

A further aspect of the present invention is the
method for treating bacterial infections by interrupting
the expression of the MMS operon by binding an antisense
10 oligonucleotide antibiotic to a mRNA transcribed from the
MMS operon.

In preferred embodiments, the antisense
oligonucleotide antibiotic can be selected from the
15 following sequences:

5' CATCCAAAGCAGTGGTAAACTGTTT 3' (AOAMMS-dnaG),
5' TCACCGATCGGCGTTTCCA 3' (AOAMMS-rpoD),
5' GGCCCCGATTTTTAGCAA 3' (AOAMMS-Eco);
5' CTTGCGTAAGCGCCGGGG 3' (AOAMMS-Sty) and
20 5' TATTCGATGCTTTAGTGC 3' (AOAMMS-Bsu).

Another aspect of the present invention is a
method for typing or identifying bacteria comprising the
steps of binding a unique intergenic antisense
oligonucleotide to a mRNA transcribed from the MMS operon
25 and then determining the amount of binding between the
species specific MMS oligonucleotide and the mRNA
transcribed from the MMS operon of a given bacterial
species.

In the treatment of a bacterial infection or in
30 the identification of bacteria the antisense
oligonucleotide is at least 10 nucleotides (10 mer). In a
preferred embodiment, an oligonucleotide of 16 to 26 mers
is used.

An additional aspect of the present invention is
35 the provision of an antisense oligonucleotide antibiotic
of at least 10 nucleotides, wherein said oligonucleotide

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1 binds to a mRNA transcribed from a MMS operon. In one
embodiment the antibiotic further comprises a carrier
molecule linked to the oligonucleotide for facilitating
5 the uptake of the oligonucleotide into the bacterium. The
carrier molecule can be an amino acid, and in one
preferred embodiment is leucine. In another embodiment
the 3' end of the oligonucleotide is derivatized to
prevent the degradation, e.g. by exonucleases, of the
10 oligonucleotide after bacteria uptake.

Other and further objects, features and
advantages will be apparent from the following description
of the presently preferred embodiments of the invention
given for the purpose of disclosure when taken in
15 conjunction with the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is the MMS operon shown in schematic
form. It contains three genes, one each, involved in the
initiation of translation (rpsU), replication (dnaG) and
20 transcription (rpoD).

Figure 2 depicts the regulation of the E. coli
MMS operon. The three genes in the MMS operon are
depicted as closed boxes. The cis-acting regulatory
sequences include promoters (P_x , P_1 , P_2 , P_3 , P_a ,
25 P_b , P_{hs}), terminators (T_1 and T_2), a LexA binding
site, nut_{eq} and an RNA processing site. The trans
acting factors are shown with arrows drawn to where they
are believed to act. The NusA protein increases rpoD gene
expression, but its site of action is unknown. Global
30 regulatory networks that interact with the MMS operon
include the SOS, heat shock and stringent response. A
functional role for orf_x has not been assigned, but the
proximity of P_x and the conservation of the orf_x
sequences in E. coli and S. typhimurium suggests a
35 possible MMS operon regulatory role. There are several
other open reading frames further upstream with no

1 assigned function and the nearest gene mapped on the E. coli chromosome is the cca gene which is 14 kb away.

5 Figure 3 is a comparison of the MMS operon in different species. The structure of the MMS operon has been determined for E. coli, S. typhimurium and B. subtilis. The genes are depicted by open boxes with the size given in base pairs (bp) including termination codon. The size of the intergenic sequences is given below. Position of promoters (P) are denoted. AOAMMS - Eco is complementary to the E. coli MMS operon rpsU-dnaG intergenic sequences. AOAMMS - Sty is complementary to the S. Typhimurium MMS operon rpsU-dnaG intergenic sequences. AOAMMS - Bsu is complementary to the B. subtilis MMS operon rpsU-dnaG intergenic sequences.

15 Figure 4 shows a 5' modified antisense oligonucleotide antibiotic containing the addition of leucine.

20 Figure 5 shows a 3' modified antisense oligonucleotide antibiotic.

25 Figure 6 shows the homologies between bacterial strains for the primase gene. The information was generated from DNA sequences in GenBank utilizing the Molecular Biology Information Resources Multialign program to optimize homology searches of protein sequence data. The data is aligned from left to right on the abscissa, the amino terminal to the carboxy terminal portions of the protein. The numbers represent the amino acid positions in the protein primary sequence. In (a) B. subtilis was compared to E. coli, while in (b) S. typhimurium was compared to E. coli, and in (c) B. subtilis is compared to S. typhimurium. In (d), the S. typhimurium and B. subtilis primase protein sequences have been aligned to the E. coli dnaG primase in the amino terminal region. Upper case letters represent aligned non-identical amino acids while lower case letters signify non-aligned amino

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1 acids. The dashes represent aligned identical bases while
the dots signify gaps. The data demonstrate that the
primase proteins are related and share homology domains
5 particularly in the amino terminal regions. The
nucleotide sequence encoding these areas of amino acid
homology are also very homologous.

Figure 7 is a picture of 1% agarose gel showing
antisense binding.

10 The drawings are not necessarily to scale and
certain features of the invention may be exaggerated in
scale or shown in schematic form in the interest of
clarity and conciseness.

DETAILED DESCRIPTION

15 It will be readily apparent to one skilled in the
art that various substitutions and modifications may be
made to the invention disclosed herein without departing
from the scope and spirit of the invention.

The macromolecular synthesis (MMS) operon
20 includes genes involved in initiating translation, rpsU
replication, dnaG, and transcription, rpoD. These genes
are contained within a single transcriptional unit,
Figures 1 and 2, and are involved in initiating synthesis
of the major information macromolecules of the cell. The
25 organization of the operon suggests that under certain
physiological conditions there is a need for coordination
of synthesis of DNA, RNA and protein in the cell and hence
a coregulation of the initiator genes. Since the
synthesis of each class of information macromolecule (DNA,
30 RNA and protein) appears to be regulated at its initiation
step, regulation of the MMS operon most likely plays a
role in regulating cell growth.

In the MMS operon cis-acting regulatory sequences
can occur within the coding regions. In gram-negative
35 bacteria these include the nut_{eg} site within the rpsU
structural gene and promoters P_a, P_b, and P_{hs} in the

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1 dnaG structural gene. Promoter P_3 of the B. subtilis
MMS operon is within this gene coding for P23. Other
5 cis-acting regulatory sequences are located in the
intergenic regions; terminator T_1 is located between
rpsU and dnaG and an RNA processing site occurs in the
dnaG-rpoD intergenic sequences. Thus, multiple cis-acting
regulatory sequences allow discoordinate regulation as
well as differential relative rates of individual gene
10 expression within this operon structure.

Codon usage can affect relative amounts of
individual gene expression. The presence of codon
preference reflects the relative concentrations of
isoaccepting tRNA species in the cell. The use of rare
15 codons provides a means to ensure low level expression of
regulatory genes. The dnaG gene contains greater than ten
times the number of rare triplet codons as other E. coli
genes and the absolute number of rare codons in the dnaG
mRNA is similar to that of other control genes (e.g. lacI,
20 trpR). Rare codons also occur in the S. typhimurium dnaG
mRNA and the dnaE gene of B. subtilis. An additional
translational regulatory mechanism operative in the MMS
operon relies on the occurrence of ribosome binding sites
with varying degrees of complementarity to the
25 Shine-Dalgarno sequence. This can be seen in the E. coli
dnaG gene, and is presumably due to the difference in free
energy of binding leading to less efficient binding of the
ribosome to the dnaG portion of the MMS mRNA. Both of
these translational regulatory mechanisms, rare codon
30 usage and altered ribosome binding affinity may partially
explain the observed apparent discoordination of
expression of the genes in this operon. The steady state
relative abundances for the MMS operon protein products in
the E. coli cell are 40,000 for S21, 50 for primase and
35 approximately 3000 for sigma-70.

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1 Comparative analysis of three sequenced MMS
operons reveals several interesting features (Figure 3).
All of the operons contain three open reading frames and
transcription of the operons is initiated by several
5 promoters at the 5' end. The major promoters have
overlapping nucleotide sequences (-10 and -35 regions) and
the cis-acting regulatory sequences appear to be clustered
in small regions. Each operon contains a heat shock
10 promoter (P_{hs}) within the DNA replication initiation
gene, dnaG or dnaE. The E. coli and S. typhimurium
operons contain an open reading frame (orf_x) upstream of
the external promoters (P_1 , P_2 , P_3). Only 7 bp
separate the -35 sequences of P_x and P_1 in E. coli
15 while these sequences actually overlap in the S.
typhimurium operon.

The central gene in the MMS operon is the one
involved in initiating DNA replication. The dnaG gene
product, primase has several activities which include (i)
20 a protein-protein interaction with the primosome complex,
(ii) a protein-nucleic acid interaction for recognition of
the origin, (iii) an RNA polymerase activity to synthesize
the primer RNA and (iv) a role in the partitioning of
chromosomes as suggested by the parB mutation in the dnaG
25 gene. There are no promoters which transcribe the dnaG
gene directly. A 5' transcription terminator, poor
ribosome binding site, occurrence of rare codons and
clustering of rare codons are all mechanisms that maintain
low level expression of this gene. Overexpression of the
30 dnaG gene from a regulated promoter on an autonomously
replicating plasmid kills the host cells. Evidence that
regulation of dnaG expression directly affects cell growth
comes from Tn5 mutagenesis data. A cloned dnaG gene with
the MMS operon promoters intact, on a multicopy plasmid
35 slows the growth rate of the host cell harboring it.
After insertion of Tn5 into the dnaG promoter regions,

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1 presumably leading to decreased dnaG gene expression,
growth rates return to control levels demonstrating that
an increased dnaG expression can affect growth. Isolation
5 of the parB mutation also suggests a direct role for dnaG
in chromosome partitioning, cell division, and therefore,
bacterial cell growth. The primase proteins encoded by
the DNA replication initiation genes from the three
sequenced MMS operons contain several regions of homology
10 (Figure 6).

The MMS operon is under very complex regulatory
control which, teleologically would be expected of a unit
whose control is important to regulation of cell growth.
In addition to the intrinsic complex regulation, the
15 operon interacts with several global regulatory networks
including heat shock, the stringent response, and SOS.
This operon appears to have evolved ways to be regulated
both as a single unit and as a group of independent units
by strategic positioning of transcriptional and
20 translational control signals. The fact that the operon
is the same in E. coli and S. typhimurium and very similar
in B. subtilis suggests there is a selective advantage to
evolving such a structure.

The term "oligonucleotide" as used herein defines
25 a molecule comprised of more than three
deoxyribonucleotides or ribonucleotides. Its exact length
will depend on many factors relating to the ultimate
function or use of the oligonucleotide.

The term "homologous sequence" as used herein
30 defines a sequence within the MMS operon which has been
conserved in bacterial species such that the sequence is
nearly identical among a variety of species. Thus, this
sequence because of its identity cannot be used to
distinguish different types of bacteria from themselves
35 but can be used as a location which can be attacked by a
single agent to interfere with a variety of bacterial
species.

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The term "unique intergenic sequences" as used herein defines a section of non-coding DNA between specific genes. In the MMS operon the intergenic sequences as seen in Figure 3 are unique for each different strain of bacteria. Thus, a specific sequence will be characteristic for a specific strain of bacteria and thus, can be used to identify the bacteria or for the specific binding an an agent to kill or interrupt the functioning of that type of bacteria only.

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The term "antisense" as used herein defines an oligonucleotide the sequence of which is complementary to the sense strand of the MMS operon. An antisense oligonucleotide will bind (form a complex by Watson-Crick base pairing) in a complementary fashion to the messenger RNA molecule which has been transcribed from the MMS operon, as well as to a single stranded DNA of the MMS operon.

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The term "antibiotic" as used herein means an oligonucleotide capable of interfering with the MMS operon to slow down bacterial growth thereby arresting growth and provoking cell death.

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"Derivitizing" the oligonucleotide means altering the structure of the oligonucleotide to perform a specific function (e.g. (1) an addition to the 5' end to afford uptake into the cell; (2) blocking the 3' end to prevent exonucleolytic breakdown). This procedure provides a more functional and stable oligonucleotide when it is in the bacteria. For example, the 3' end can be derivitized by adding a phosphorothioate linked nucleotide.

35

In one embodiment of the present invention there is included a method of interrupting the expression of a MMS operon comprising the step of binding antisense oligonucleotide to an mRNA transcribed from the MMS operon. In this method the antisense oligonucleotide binds to the mRNA which is transcribed from the MMS

1 operon. After the binding of the antisense
oligonucleotide the mRNA is unable to be translated into
the proteins encoded by the MMS operon. In order to
5 inactivate the mRNA, only a small segment of the mRNA must
be bound to the antisense oligonucleotide.

The antisense oligonucleotide is selected from
the group consisting of a sequence specific to a unique
intergenic sequence, a sequence specific to a bacterial
10 homologous expressed sequence and any combination
thereof.

By binding to a specific unique intergenic
sequence encoded in the single stranded DNA or mRNA which
has been transcribed from the MMS operon, the antibiotic
15 can be targeted to interrupt and kill the specific type of
bacteria. By binding to the homologous sequence, the
antibiotic can be targeted to a wide variety of bacteria
all containing the homologous sequence. Depending on the
length of the oligonucleotide or the location of the mRNA
20 which is bound, the oligonucleotide may overlap and bind
to both a unique sequence and a homologous sequence.

Although the length of the oligonucleotide which
is necessary to inhibit the functioning of the mRNA is
unknown, it should be at least 10 nucleotides (10 mer).
25 In one embodiment of the present invention, the
oligonucleotide is in the range of 16 to 26 mers.

An additional aspect of the present invention is
a method for treating bacterial infections comprising the
step of interrupting the expression of a MMS operon by
30 binding an antisense oligonucleotide antibiotic to a mRNA
transcribed from said MMS operon. The antisense
oligonucleotide antibiotic can bind to either a homologous
sequence, a unique intergenic sequence or a combination
thereof. Some examples of sequences which can be used to
35 bind to the mRNA to interrupt the function of the MMS
operon and thus to treat bacterial infections are seen in
Table 1.

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Table 1

Sequences which bind to mRNA transcribed
from the MMS operon

- (1) 5'CATCCAAAGCAGTGGTAAACTGTTT 3' (AOAMMS-dnaG),
- (2) 5'TCACCGATCGGCGTTTCCA 3' (AOAMMS-rpoD),
- (3) 5' GGCCCCGATTTTTAGCAA 3' (AOAMMS-Eco),
- (4) 5' CTTGCGTAAGCGCCGGGG 3' (AOAMMS-Sty),
- (5) 5' TATTCGATGCTTTAGTGC 3' (AOAMMS-Bsu).

The first two sequences (1-2) bind to bacterial homologous sequences and thus are not specific to any type of bacteria. These sequences can be used to treat a wide class of bacterial infections since they attack both gram positive and gram negative bacteria. The last three sequences (3-5) are unique intergenic sequences which bind to specific sequences in specific bacteria. For example sequence (3) is specific to E. coli. Thus, employing this antisense oligonucleotide antibiotic will specifically inhibit the MMS operon of E. coli while not attacking the MMS operon of other bacteria. Sequence (4) specifically binds the transcribed mRNA of S. typhimurium and sequence (5) specifically binds the mRNA of B. subtilis. Thus, by employing the antisense oligonucleotide antibiotics (3-5) a specific antibiotic can be used to kill a specific bacteria. Thus, the treatment to kill or interfere with the reproduction of specific bacterial strains can be targeted.

In the preferred embodiment, using unique sequences, the nucleotide sequence of the proposed antisense oligonucleotide antibiotics is complementary to the intergenic region of the 5' side of the DNA replication initiation gene (dnaG or dnaE) (see Figure 3). This region of the MMS operon is chosen because the replication initiation gene has the lowest level of expression within the operon. Furthermore, in E. coli and S. typhimurium, this gene is located downstream from a

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1 terminator and is not directly transcribed by any
promoter. In order to provide a more stable interaction
with the mRNA the primary sequences of the antisense
5 oligonucleotide antibiotic are chosen to maximize GC base
pairing. However, there is usually a balance between
maintaining the uniqueness of the sequence and maximizing
the GC base pairing.

10 Another embodiment of the invention is a method
of identifying bacteria comprising the steps of binding a
unique species specific intergenic antisense
oligonucleotide to a mRNA transcribed from a MMS operon of
a given species and determining the amount of said
binding. The unique sequence will only bind to a specific
15 bacteria strain, therefore no binding indicates a
different strain and binding indicates the strain with the
specific sequence. Each bacteria strain contains its own
unique intergenic sequence which can be used to uniquely
identify each strain. The mRNA which is transcribed from
20 the MMS operon spans the whole operon and contains the
unique intergenic sequence. By designing oligonucleotides
which bind to these unique sequences, the diagnosis and
treatment can be tailored to only interfere with the
functioning of a MMS operon in those bacteria strains
25 which have that unique sequence. Thus, by using a variety
of antisense oligonucleotide probes, bacteria can be typed
for each individual strain. The amount of binding can be
determined by a variety of methods known to those skilled
in the art, including radioisotopes, enzymes, fluorescers,
30 antibodies and chemiluminescers. For example, the unique
species specific intergenic antisense oligonucleotides can
be labelled with biotin and then identified by a Strep
avidin complex or a fluorescent tag.

35 For example, the antisense oligonucleotide of
sequence (3) table 1 can be used to identify E. coli,
whereas the antisense oligonucleotide of sequence (4)

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1 table 1 can be used to identify S. typhimurium and the
antisense oligonucleotide of sequence (5) table 1 can be
used to identify B. subtilis. One skilled in the art will
5 readily recognize that as additional MMS operon intergenic
sequences are sequenced additional bacteria can be
identified by antisense oligonucleotides synthesized to
the unique intergenic sequences.

10 In bacteria typing the length of the antisense
oligonucleotide will be determined by the size necessary
to bind specifically to the unique sequence. The
oligonucleotide will be at least 10 nucleotides. In one
preferred embodiment the sequences are between 16 and
26 mers. Examples of some preferred sequences are found
15 in table 1 sequences (3-5).

In order for the antisense oligonucleotide
antibiotic to effectively interrupt the MMS operon
function by binding to the mRNA transcribed from the MMS
operon, the antisense oligonucleotide antibiotic must
20 enter the bacterial cell. Although some oligonucleotides
can be taken up by certain bacterial cells (e.g.
Haemophilus), other oligonucleotides will need to be
modified to facilitate uptake. Thus, it may be necessary
to link a carrier molecule, for example an amino acid, to
25 the oligonucleotide. In Figure 4, the oligonucleotide is
modified at the 5' end by adding a leucine molecule to the
oligonucleotide. Bacteria have multiple transport systems
for the recognition and uptake of molecules of leucine.
The addition of this amino acid to the oligonucleotide
30 will facilitate the uptake of the oligonucleotide in the
bacteria and will not interfere with the binding of the
antisense oligonucleotide to the mRNA molecule.

One skilled in the art will readily recognize
that other methods are available for facilitating the
35 uptake of the antisense oligonucleotide antibiotic in the
bacteria. For example, addition of other amino acids will

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1 enable utilization of specific amino acid transport
systems. Addition of lactose to the oligonucleotide by a
covalent linkage may enable transport by lactose permease
5 (product of the lac operon Y gene). Other sugar transport
systems, known to be functional in bacteria, can be
utilized to facilitate uptake into the bacterial cell.

Once an oligonucleotide with or without the
carrier has entered the bacterial cell, it is important
10 that it remain stable for the time period necessary to
bind to the mRNA transcribed by the MMS operon. In one
embodiment of the present invention, the oligonucleotide
is derivatized at the 3' end to prevent degradation of the
oligonucleotide (Figure 5). Other methods are known to
15 alter the 3' and/or 5' ends of oligonucleotides to prolong
the intracellular life and thus increase the availability
for binding to the mRNA.

In addition to interrupting the MMS operon by
binding to the mRNA transcribed from the operon, it is
20 also possible to control other downstream products of the
MMS operon to interrupt bacteria and to treat bacterial
infections. For example, interrupting the function of the
proteins encoded in the MMS operon will also interrupt the
function of the MMS operon and lead to death of the
25 bacteria.

One embodiment of the present invention is a
method for treating bacterial infections comprising the
step of interrupting the function of proteins selected
from the group consisting of S21, primase and sigma-70.
30 This method comprises the step of competitively inhibiting
a recognition site of a protein encoded by the MMS operon
by introducing a competitive oligonucleotide into the
bacteria.

The S21 recognition site includes the
35 Shine-Dalagarno sequence located at the 3' end of the 16S
rRNA and may be inhibited by introducing an

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1 oligonucleotide which competitively inhibits the binding
of S21 in the bacteria. For example, an oligonucleotide
of the sequence 5'GATCACCTCCTTA 3' which is the 3' end of
5 the 16S rRNA (the Shine-Dalagarno sequence).

The primase recognition site includes the phage
G4 origin of replication site. Thus by introducing into
bacteria a competitive oligonucleotide which interferes
with this recognition site, bacterial growth and survival
10 may be inhibited. An example of this competitive
inhibitor is

5'GGCCGCCCCACATTGGGCAGGTATCTGACCAGTAGAGGGGCGGCC 3' which
is the loop III of the bacteriophage G4 ori_c.

The sigma-70 recognition site includes the core
15 polymerase α_2 BB' and this interaction confers
specificity for promoter sequences. An example of this
competitive inhibitor is 5'TTGACATAAATACCACTGGCGGTGATACT
3'. This sequence is the bacteriophage lambda P_L
promoter. This is the strongest promoter in E. coli and
thus has the strongest known binding with RNA polymerase.
20

Thus the introduction of competitive
oligonucleotides for these sequences into the bacteria
will result in competitive interaction with the protein
recognition site, thus preventing the binding of the S21,
25 primase or sigma-70 molecules to the recognition site.
This will interrupt normal cell function, growth and
replication. Introduction of these oligonucleotides into
the bacteria, disrupts the MMS operon's function and thus
successfully treats bacterial infections.

Example I

30 To inhibit cell growth, an inoculum of E. coli
and B. subtilis are mixed in a single test tube and an
antisense oligonucleotide to E. coli (AOAMMS-Eco) is added
to the cell inoculum. The culture is gram strained after
35 several hours of growth. Gram positive organisms are seen
and there is a paucity of gram negative organisms. In a

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1 corollary experiment, an antisense oligonucleotide to B. subtilis (AOAMMS-Bsu) is added to a mixed inoculum of E. coli and B. subtilis and it is grown for several hours. On
5 subsequent gram stain there is found negative rods. These experiments demonstrate species specific antisense oligonucleotide demise of bacterial organisms.

EXAMPLE II

To show that the expressed sequences within the
10 MMS operon (rpsU, dnaG, rpoD) contain conserved homologous DNA sequences, the following oligonucleotide which recognized conserved DNA sequences within the dnaG gene.

AOAMMS - dnaG, 5'- CATCCAAAGCAGTGGTAAACTGTTT-3' was synthesized: (sequence 1, Table 1)

15 This oligonucleotide was end labeled and used as a probe in Southern blotting. DNA was isolated from 12 different pathogenic strains of Salmonella obtained from the body fluids of infected patients, digested with HindIII and run on a 1% agarose gel. This digested
20 chromosomal DNA was probed with the end-labeled dnaG oligonucleotide AOAMMS.

As seen in Figure 7, there is conservation of the oligonucleotide AOAMMS - dnaG in different pathogenic strains of Salmonella. The Southern blot shows homology
25 of the oligonucleotide AOAMMS-dnaG to a laboratory control strain of Salmonella (LT-2) (lane 1) and twelve (12) different pathogenic strains isolated from body fluids of patients (lanes 2-13). There was no hybridization to human DNA (the negative control on lane 14), and as a
30 positive control; a plasmid containing the DNA sequences in the probe showed a hybridization signal (lane 16). Lane 15 has lambda DNA cut with Hind III as a marker. On the far right are the sizes in kilobase pairs as determined on the agarose gel before Southern transfer.

35 One skilled in the art will readily appreciate that the present invention is well adapted to carry out

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1 the objects and obtain the ends and advantages mentioned,
as well as, those inherent therein. The oligonucleotides,
antibiotics, compounds, methods, procedures and techniques
5 described herein are presently representative of preferred
embodiments, are intended to be exemplary, and are not
intended as limitations on the scope. Changes therein and
other uses will occur to those skilled in the art which
are encompassed within the spirit of the invention or
10 defined by the scope of the appended claims.

WHAT IS CLAIMED IS:

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CLAIMS

1. A method of interrupting the expression of a MMS operon, comprising the step of binding an antisense oligonucleotide to a mRNA transcribed from said MMS operon.

2. The method of claim 1, wherein the antisense oligonucleotide is selected from the group consisting of a sequence specific to a unique intergenic sequence, a sequence specific to a bacterial homologous sequence and any combination thereof.

3. The method of claim 2, wherein the antisense oligonucleotide is at least 10 mers.

4. The method of claim 3, wherein the antisense oligonucleotide is 16 to 26 mers.

5. A method for treating bacterial infections comprising the step of interrupting the expression of a MMS operon by binding an antisense oligonucleotide antibiotic to a mRNA transcribed from said MMS operon.

6. The method of claim 5, wherein the antisense oligonucleotide antibiotic binds to a bacterial homologous sequence in the mRNA transcribed from said MMS operon.

7. The method of claim 6, wherein the antisense oligonucleotide antibiotic is selected from the group consisting of 5'-CATCCAAAGCAGTGGTAAACTGTTT 3' and 5'-TCACCGATCGGCGTTTCCA 3'.

8. The method of claim 5, wherein the antisense oligonucleotide antibiotic binds to an intergenic sequence, said intergenic sequence is unique for each strain of bacteria.

9. The method of claim 8, wherein the antisense oligonucleotide antibiotic is selected from the group consisting of:

5' GGCCCCGATTTTGTAGCAA 3' which binds to the transcribed mRNA of E. coli, 5' CTTGCGTAAGCGCCGGG 3' which binds to the transcribed mRNA of S. typhimurium,

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1 and 5' TATTCGATGCTTTAGTGC 3' which binds to the
transcribed mRNA of B. subtilis.

5 10. The method of claim 5, wherein the antisense
oligonucleotide antibiotic binds to both a homologous
sequence and a unique intergenic sequence.

11. The method of identifying bacteria,
comprising the steps of:

10 binding a unique intergenic antisense
oligonucleotide to a mRNA transcribed from a MMS
operon; and

determining the amount of said binding.

12. The method of claim 11, wherein the
oligonucleotide is:

15 5' GGCCCCGATTTT TAGCAA 3' and the bacteria is
identified as E. coli.

13. The method of claim 11, wherein the
oligonucleotide is:

20 5' CTTGCGTAAGCGCCGGGG 3' and the bacteria is
identified as S. typhimurium.

14. The method of claim 11, wherein the
oligonucleotide is

5' TATTCGATGCTTTAGTGC 3' and the bacteria is
identified as B. subtilis.

25 15. An antibiotic, comprising:

at least a 10 mer oligonucleotide, wherein said
oligonucleotide is complementary to a sense strand of
a MMS operon and binds to a mRNA transcribed by said
sense strand.

30 16. The antibiotic of claim 15, wherein said
oligonucleotide is selected from the group consisting of:

5'GGCCCCGATTTT TAGCAA 3', 5'CTTGCGTAAGCGCCGGGG 3',

5'TATTCGATGCTTTAGTGC 3',

5'CATCCAAAGCAGTGGTAAA ACTGTTT 3', and

35 5'TCACCGATCGGCGTTTCCA 3'.

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1 17. The antibiotic of claim 15, further comprising:

5 a carrier molecule linked to said oligonucleotide, wherein said carrier molecule facilitates the uptake of said oligonucleotide into the bacterium.

18. The antibiotic of claim 17, wherein the carrier molecule is an amino acid.

10 19. The antibiotic of claim 15, wherein said oligonucleotide is derivatized at the 3' end to prevent degradation of said oligonucleotide.

15 20. The antibiotic of claim 19 wherein a phosphorothioate linked nucleotide is added to the 3' end by derivatization.

21. A method of treating bacterial infections, comprising the step of interrupting the function of proteins selected from the group consisting of S21, primase and sigma-70.

20 22. The method of treating bacterial infections, comprising the step of competitively inhibiting a recognition site of a protein encoded by a MMS operon by introducing a competitive oligonucleotide into a bacterium.

25 23. The method of claim 22, wherein a S21 recognition site is inhibited by introducing 5'GATCACCTCCTTA 3' into the bacterium.

30 24. The method of claim 22, wherein a primase recognition site is inhibited by introducing 5'GGCCGCCCCACATTGGGCAGGTATCTGACCAGTAGAGGGCGGCC 3' into the bacterium.

25. The method of claim 22, wherein a sigma-70 recognition site is inhibited by introducing 5'TTGACATAAATACCACTGGCGGTGATACT 3' into the bacterium.

35 26. The method of identifying bacteria, comprising the steps of:
treating a MMS operon to form single stranded DNA;

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1 binding an antisense oligonucleotide to a unique
intergenic sequence in the single stranded DNA of the
MMS operon; and

5 measuring the amount of said binding.

27. The method of claim 26, wherein the
oligonucleotide is:

10 '5 GGCCCCGATTTT TAGCAA 3' and the bacteria is
identified as E. coli.

28. The method of claim 26, wherein the
oligonucleotide is:

15 '5 CTTGCGTAAGCGCCGGGG 3' and the bacteria is
identified as S. typhimurium.

29. The method of claim 26, wherein the
oligonucleotide is:

20 '5 TATTCGATGCTTTAGTGC 3' and the bacteria is
identified as B. subtilis.

25

30

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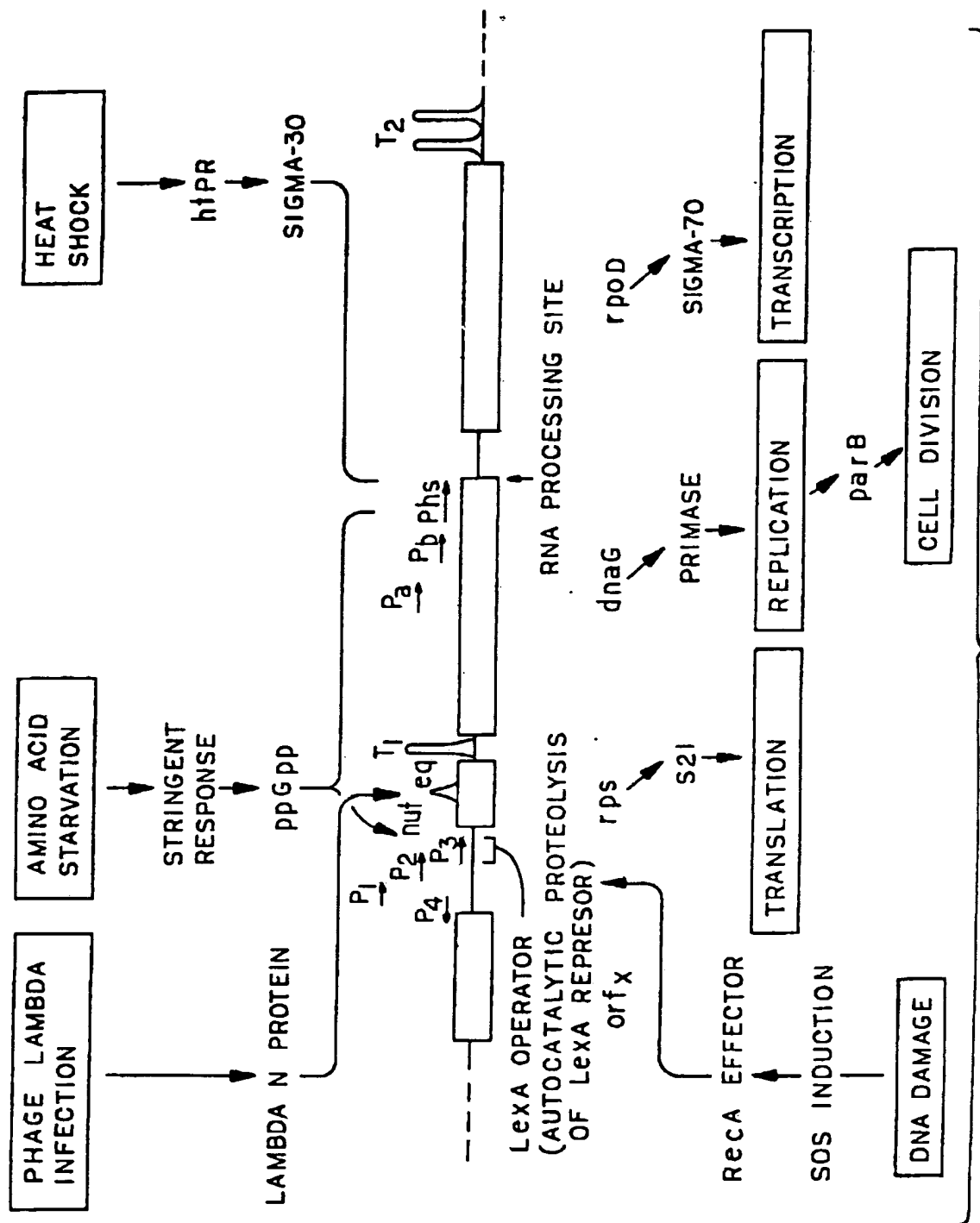


FIG. 2

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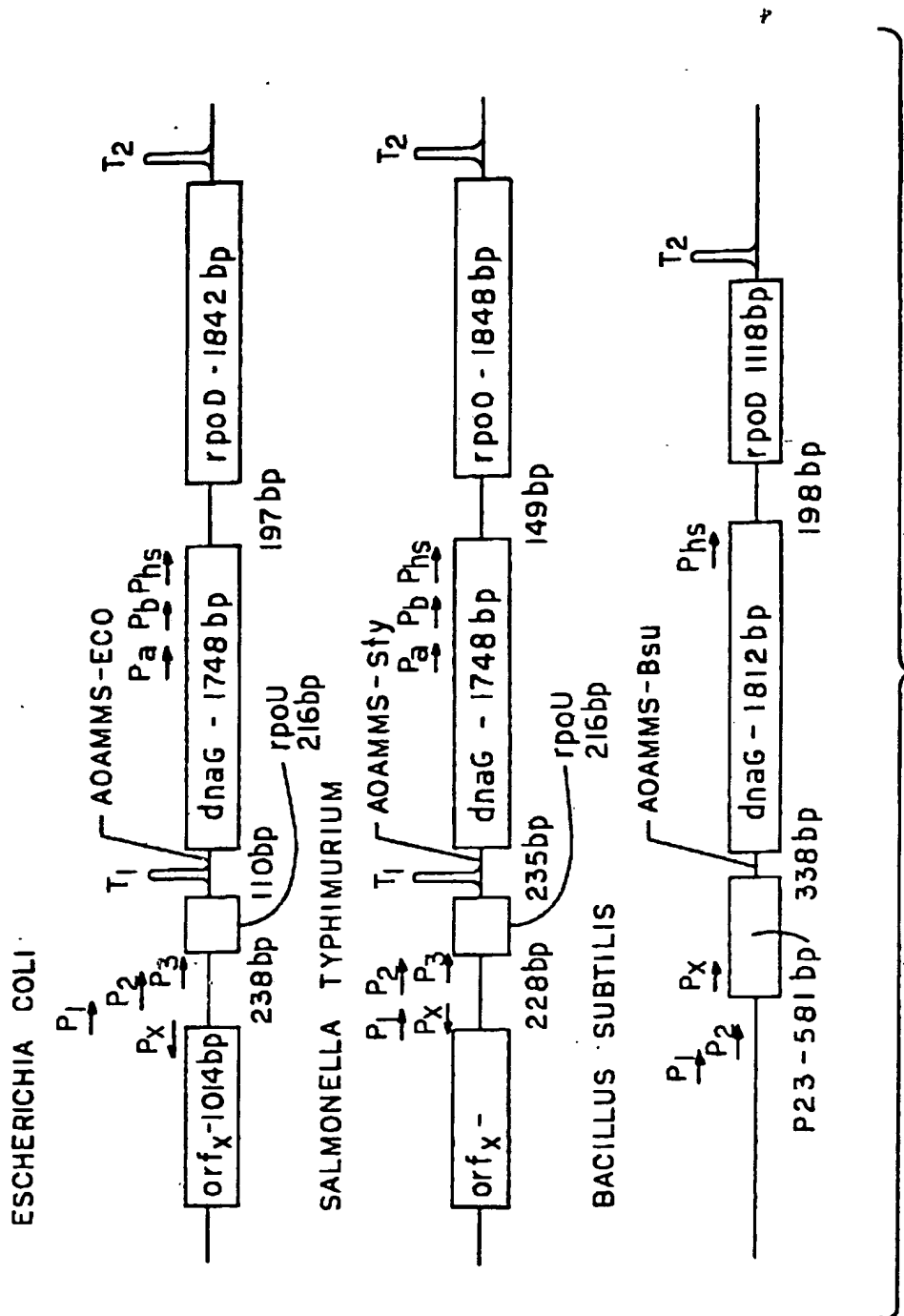


FIG. 3

SEQUENCE OF THE

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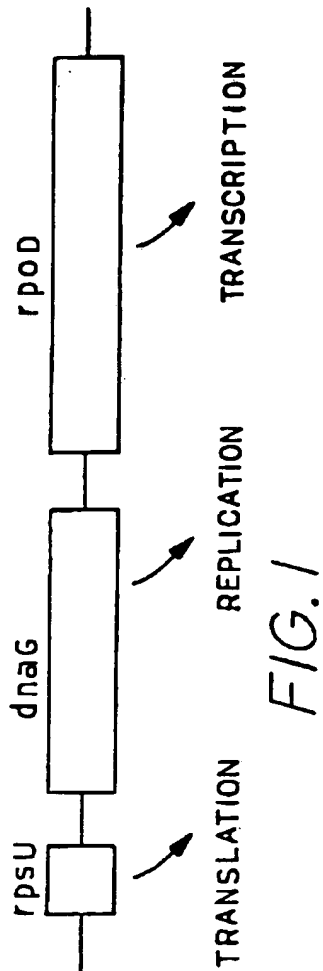


FIG. 1

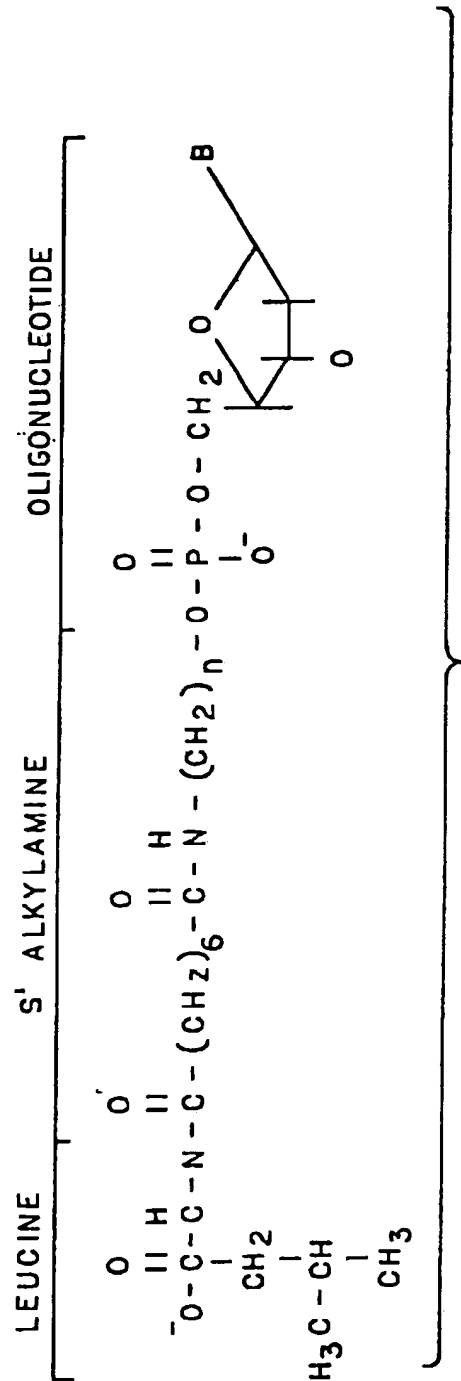


FIG. 4

SUBSTITUTE SHEET

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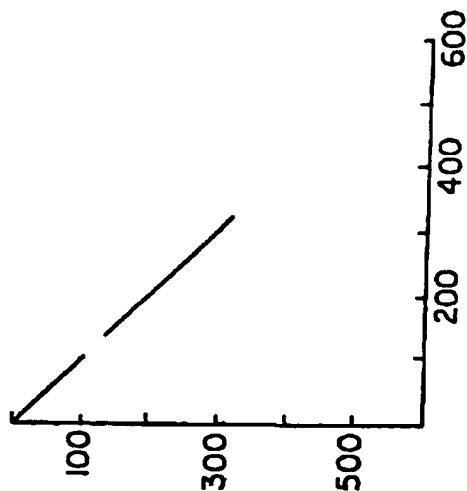
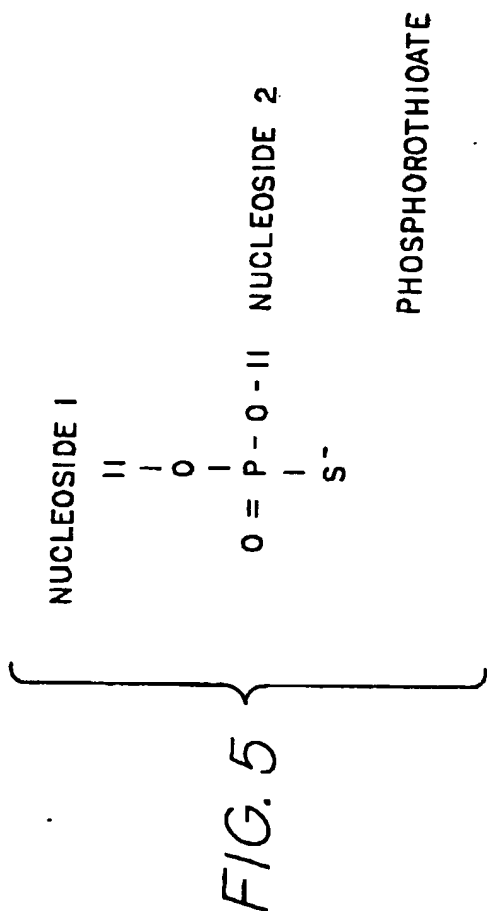


FIG. 6A

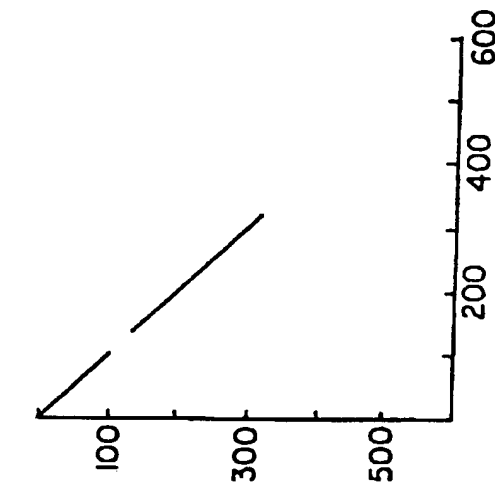


FIG. 6C

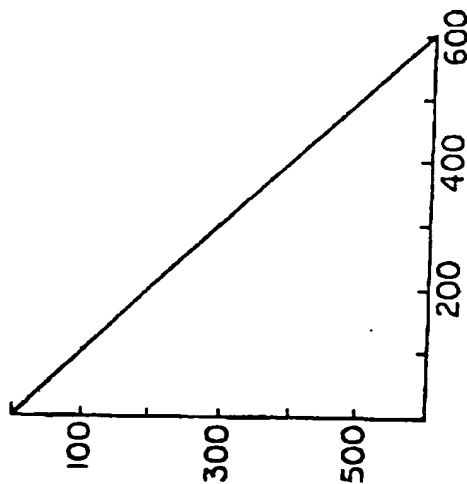


FIG. 6B

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E. COLI	:	MAGRIPRVFINDLLARTDIVDLIDARVKKKQGNFACCPFHNEKTPSFTVNGEKQFYH
S. TYPHYMURIUM	:	-----V-----Y-----
B. SUBTILIS	:	-GN---DEIVDQVQKSA---EV-GDY-Q-----R YFGL-----G S-----S-SPD--IF-
E. COLI	:	CFGCGAHGNAIDFLMNYDKLEFVETVEELAAMHNLEVPFE . AGSGPSQIERHQRQTLYQL
S. TYPHYMURIUM	:	-----I-Y-.-T-L-----N-----
B. SUBTILIS	:	-----G--VFS--RQMEGYS-A-S-SH--DKYQIDF-DDITVHSGARP-SSGE-KMAEA
E. COLI	:	MDGLNTFYQQSL.QQPVATSARQYLEKRGLSHEVIARFAIGFAPPGWDNVLKRFGGNPEN
S. TYPHYMURIUM	:	-N---D-----TH-A-KP--D--Q-----A-I-Q-----A-----N-SD-
B. SUBTILIS	:	HEL-KK--HHL-1NTKEGQE-LD--LS--FTK-L-NE-Q--Y-LDS--FIT-FLV KRGFS
E. COLI	:	RQSLIDAGMLVTNDQGRSY.DRFRERVMFPI RDKRGRVIGFGRVLGNDTPKYLNSPETD
S. TYPHYMURIUM	:	KAL-L-----N-E--ST-.-N-----
B. SUBTILIS	:	EAQMEK--L-IRRED-SG-f---N-----H-HH-A-VA-S-A--SQQ---M-----P
E. COLI	:	IFHKGRQLYGLYEAQQDNAEPNRLLVVEGYMDVVALAQYGINYAVASLGSTTADHIQLL
S. TYPHYMURIUM	:	-----YS---Q-----D-----MHM-
B. SUBTILIS	:	L---SKL--NF-K-RLHIRKQE-AVLF--FA--YTA VSSDVKESI-TM---L-D--VKI-
E. COLI	:	FRATNNVICCYGDGRAGRDAAWRA
S. TYPHYMURIUM	:	-----
B. SUBTILIS	:	R-NVEEI-L---S-K--YE-TLK-

FIG. 6D

SUBSTITUTE SHEET

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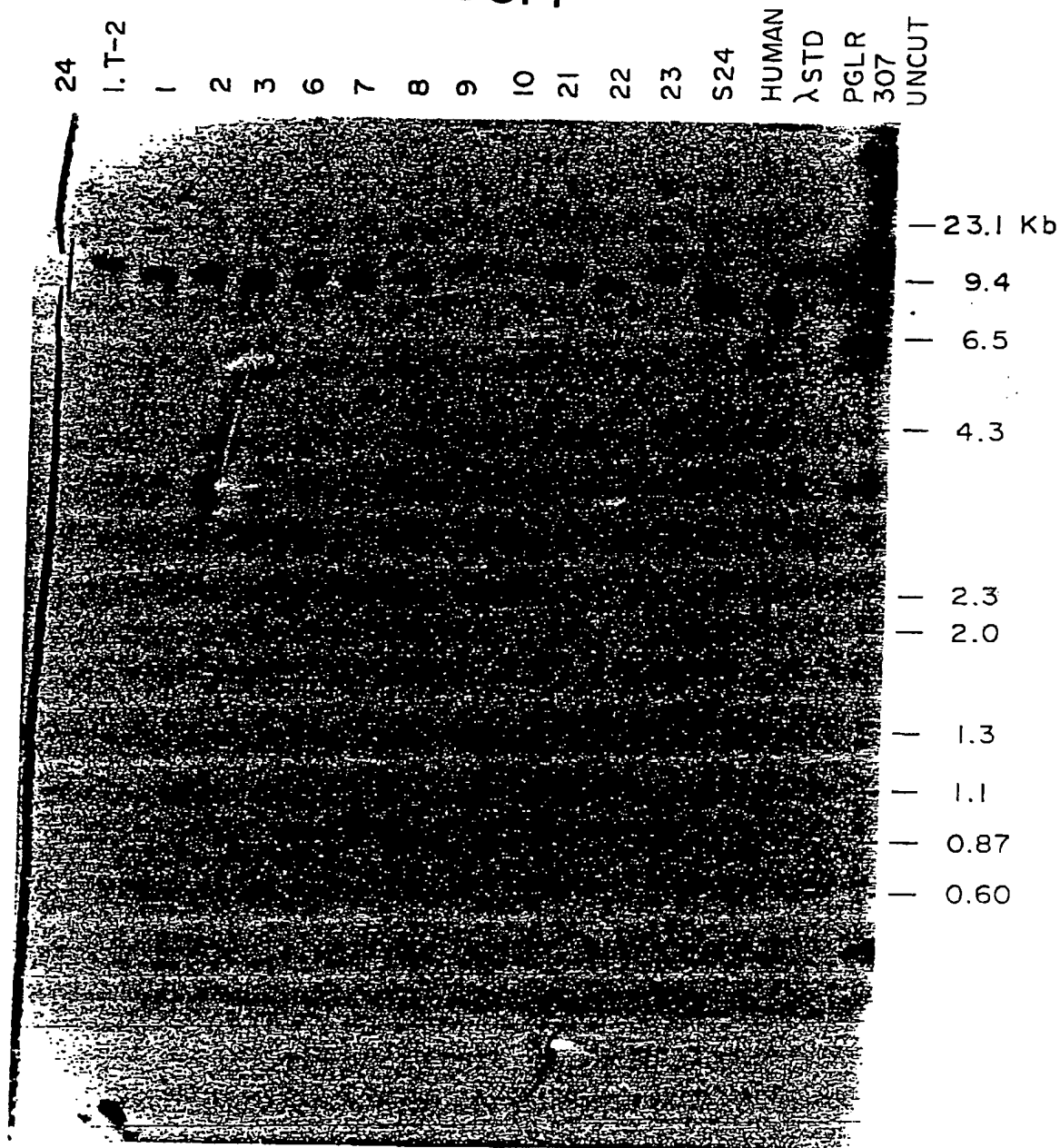


FIG. 7

SUBSTITUTE SHEET

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US89/02884

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶

According to International Patent Classification (IPC) or to both National Classification and IPC
 IPC(4): C 12 Q 1/68; C 12 N 15/00; C 07 H 15/12
 U.S. Cl: 435/6, 172.3; 514/44; 536/27

II. FIELDS SEARCHED

Minimum Documentation Searched ⁷

Classification System	Classification Symbols
U.S.	435/6, 172.3; 935/5, 6, 8, 34, 44, 72, 78; 514/44 536/27

Documentation Searched other than Minimum Documentation

⁸
 In the extent that such Documents are included in the Fields Searched ⁶
 Chemical Abstracts Data Base (CAS) 1967-1989; Biological
 Abstracts Data Base (BIOSIS) 1967-1989; MEDLINE 1967-1989.
 KEYWORDS: ANTISENSE, MESSAGE, MESSENGER, MRNA.

III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹

Category ⁹	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
Y,P	US, A, 4,801,540 (HIATT ET AL) 31 JANUARY 1989, see entire document, particularly columns 9-11.	1-29
Y	US, A, 4,740,463 (WEINBERG ET AL) 26 APRIL 1988, see entire document, particularly columns 5 and 6.	1-29
Y	US, A, 4,358,535 (FALKOW ET AL) 9 NOVEMBER 1982, see entire document, particularly columns 2-5.	11-14 & 26-29
Y	JOURNAL OF BACTERIOLOGY, Volume 169, No. 7, issued 30 June 1987 (AIBA ET AL) "Function of micF as an antisense RNA in osmoregulatory expression of the ompF gene in Escherichia coli". See entire document, particularly pages 3007-3008.	1-29

¹⁰ Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search

03 NOVEMBER 1989

Date of Mailing of this International Search Report

21 NOV 1989

International Searching Authority
 ISA/US

Signature of Authorized Officer
 THOMAS D. MYS

Thomas D. MYS

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y	NUCLEIC ACIDS RESEARCH, Volume 14, No. 10, issued 1986 (WANG ET AL) "Nucleotide sequence and organization of Bacillus subtilis RNA polymerase major sigma operon". See entire document, particularly pages 4293-4295.	1-29
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES (USA), Volume 80, issued February 1983 (KONIGSBERG ET AL) "Evidence for use of rare codons in the dnaG gene and other regulatory genes of Escherichia coli". See entire document, particularly pages 687-689.	1-29

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE ¹

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers _____, because they relate to subject matter ¹² not required to be searched by this Authority, namely:

2. ☐ Claim numbers _____, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out ¹³, specifically:

3. ☐ Claim numbers _____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING ²

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	GENE, Volume 40, issued 1985, (ERICKSON ET AL) "Nucleotide sequence of the rpsU-dnaG-rpoD operon from Salmonella typhimurium and a comparison of this sequence with the homologous operon of Escherichia coli". See entire document, particularly pages 67-69.	1-29
Y	CELL, Volume 42, issued August 1985, (KIM ET AL) "Stable reduction of thymidine kinase activity in cells expressing high levels of anti-sense RNA". See entire document, particularly, pages 129-131.	1-29
Y	CELL, Volume 39, issued December 1984, (LUPSKI ET AL) "The rpsU-dnaG-rpoD macromolecular synthesis operon of E. coli". See entire document, pages 251-252.	1-29
Y	TRENDS IN BIOCHEMICAL SCIENCE, Volume 9, No. 11, issued November 1984, (LAPORTE) "Anti-sense RNA: a new mechanism for the control of gene expression". See entire document, page 463.	1-29
Y	MOLECULAR AND GENERAL GENETICS (MGG), Volume 195, issued 1984, (LUPSKI ET AL) "Promotion, termination, and anti-termination in the rpsU - dnaG-rpoD macromolecular synthesis operon of E. coli K-12". See entire document, particularly pages 391-393.	1-29
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES (USA), Volume 80, issued July 1983, (LEARY ET AL) "Rapid and sensitive colorimetric method for visualizing biotin-labeled DNA probes hybridized to DNA or RNA immobilized on nitrocellulose: Bio-blots". See entire document, pages 4045-4049.	1-29

Form PCT/ISA/210 (and sheets) (Rev.11-87)